Biochem. J. (1983) **211**, 243–250
Printed in Great Britain

Use of procainamide gels in the purification of human and horse serum cholinesterases

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(*Received 7 October 1982/Accepted 29 October 1982*)

Two large-scale methods based primarily on the use of procainamide–Sepharose gels were developed for the purification of horse and human serum non-specific cholinesterases. With method I, the procainamide–Sepharose 4B gel was used in the first step to handle large volumes of serum. With method II, the procainamide–Sepharose 4B gel was used in the final step to obtain pure enzyme. Although both methods gave electrophoretically pure cholinesterase preparations in good yields, they were significantly more efficient at purifying the horse enzyme than the human enzyme. To study this problem, the relative binding of human and horse cholinesterases to procainamide–, methylacridinium (MAC)–, *m*-trimethylammoniophenyl (*m*-PTA)– and *p*-trimethylammoniophenyl (*p*-PTA)–Sepharose 4B gels were measured, by using two approaches. In one, binding was measured by a procedure involving equilibration of pure cholinesterase in a small volume of diluted gel slurry (4%, v/v). A partially purified preparation of *Electrophorus* acetylcholinesterase was included. Pure human cholinesterase bound consistently more tightly to each of the gels than did horse cholinesterase, and the acetylcholesterase appeared to bind the gels 10–100 times more tightly than did the non-specific cholinesterases. The order of binding for the cholinesterases, beginning with the tightest, was: procainamide–Sepharose 4B, MAC–Sepharose 4B, *p*-PTA–Sepharose 4B and *m*-PTA–Sepharose 4B. For the acetylcholinesterase the order was: MAC–Sepharose 4B, procainamide–Sepharose 4B, *p*-PTA–Sepharose 4B and *m*-PTA–Sepharose 4B. The second approach involved passing native sera or partially purified sera fractions through 1 ml test columns of each of the four affinity gels to determine their retention capacity for the cholinesterases. With these impure samples, the MAC–Sepharose 4B gels proved superior to the procainamide–Sepharose 4B gels at retaining human cholinesterase, but the opposite was true for the horse cholinesterase.

Affinity gels containing procainamide ligand were introduced by Lockridge & La Du (1978) in the final step of a small-scale purification of human serum non-specific cholinesterase (BuChE) based on the method of Das & Liddell (1970). Procainamide appears to have been the first affinity gel ligand to be used successfully in the purification of a BuChE, although several affinity gels, including gels containing *m*-PTA, *p*-PTA and MAC ligands (Kalderon *et al.*, 1970; Dudai *et al.*, 1972*a*,*b*), have been used in the purification of electric-eel acetylcholinesterase (AcChE). In the present work, two large-scale methods applicable to the purification of horse and human serum BuChE enzymes are described.

Abbreviations used: BuChE, cholinesterase (EC 3.1.1.8); AcChE, acetylcholinesterase (EC 3.1.1.7); PTA, trimethylammoniophenyl; MAC, methylacridinium.

The methods gave good yields of both enzymes, but they were significantly more efficient at purifying horse BuChE than human BuChE. The possibility of using affinity gels containing MAC, *m*-PTA or *p*-PTA ligands as alternatives to the affinity gels containing procainamide ligand for the purification of human serum BuChE was therefore explored on a small scale.

The ability of affinity gels to bind pure cholinesterases is one of the factors determining their usefulness in purifying these enzymes. Knowledge of such binding might contribute to a better understanding of the causes underlying the relatively poor efficiency of the affinity gels containing procainamide ligand at purifying human BuChE. With this in mind, one of us (A. R. M.) developed a procedure to determine the relative tightness of binding of the

two purified BuChE enzymes to each of the four affinity gels. A partially purified preparation of electric-eel AcChE was included in the study.

Experimental

Materials

Frozen non-sterile non-filtered horse serum was from Grand Island Biological Co., Grand Island, NY, U.S.A. Frozen outdated human plasma was from Rex Hospital, Raleigh, NC, U.S.A. Electric-eel AcChE was partially purified type V-S from Sigma Chemical Co., St. Louis, MO, U.S.A.

Butyrylthiocholine iodide, acetylthiocholine iodide, *NN*-dimethyl-*p*-phenylenediamine dihydrochloride, 1,2-diaminopropane and 9-chloroacridine were from Eastman Kodak Co., Rochester, NY, U.S.A. Procainamide hydrochloride, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride, 5,5′-dithiobis-(2-nitrobenzoic acid), di-isopropyl phosphorofluoridate and Paraoxon (diethyl 4-nitrophenyl phosphate) were from Sigma Chemical Co. CNBr, 6-aminohexanoic acid, 1,6-diaminohexane and 3-dimethylaminobenzoic acid were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Methyl iodide and 30–32% (w/v) HBr in acetic acid were from Fisher Chemical Co., Pittsburgh, PA, U.S.A. DEAE-Sephadex A-50 was from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Electrophoresis reagents were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Other chemicals and reagents were from the usual commercial sources.

Determination of BuChE activities

Activities were determined by the method of Ellman *et al.* (1961) as previously described (Main *et al.*, 1974). One activity unit was the hydrolysis of 1 μmol of 1 mM-substrate/min at 25°C in 0.10 M-sodium phosphate buffer, pH 8.0.

Protein concentration

Protein concentrations were routinely determined by absorbance at 280 nm. An absorption coefficient of $1\,mg^{-1}\cdot ml\cdot cm^{-1}$ was assumed unless more accurate values were available.

Preparation of affinity gels

Procainamide–Sepharose 4B affinity gel was synthesized by using the general method of Cuatrecasas & Anfinsen (1971). Typically, a 200 ml batch of washed and settled Sepharose 4B was treated at one time. The Sepharose 4B was activated wtih 8.0 g of CNBr (40 mg/ml of gel) and then coupled with 6-aminohexanoic acid (2.60 g) in 0.2 M-Na_2CO_3/$NaHCO_3$ buffer, pH 9.0, containing 0.4 M-NaCl at 5°C, to make the CH–Sepharose 4B. Procainamide (5.44 g; 100 μmol/ml of gel) was coupled to the CH–Sepharose 4B by a 0.1 M solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride by maintaining the pH at 4.5 with 1 M-HCl for 2 h and then stirring at room temperature (20–25°C) for 24 h. The concentration of the procainamide bound to the gel was determined by measuring the absorbance of the procainamide in the washings by using an absorption coefficient of $16\,150\,M^{-1}\cdot cm^{-1}$ at 278 nm. The difference between the starting amount and the amount in the washings gave the gel-bound concentration. The ligand concentrations of the other affinity gels were similarly determined by using the absorption coefficient of the appropriate compound.

The *m*-PTA–Sepharose 4B affinity gel was synthesized by coupling one of the amino groups of 1,6-diaminohexane to CNBr-activated Sepharose 4B and then coupling 3-trimethylammoniobenzoic acid to the free amino group on the hexane arm by the carbodi-imide procedure described above. The 3-trimethylammoniobenzoic acid iodide was prepared from dimethylaminobenzoic acid by refluxing 10 g with 17 g of methyl iodide in 100 ml of acetone over a steam bath for 5 h. The product was recrystallized from 95% ethanol at room temperature. The yield was 13.6 g (73%), and the m.p. was 189–191°C. An absorption coefficient of $19\,910\,M^{-1}\cdot cm^{-1}$ at 224.5 nm for the 3-trimethylammoniobenzoic acid iodide in distilled water was used to determine the ligand concentration. Literature values for this compound could not be found.

The *p*-PTA–Sepharose 4B affinity gel was synthesized by the procedure of Dudai *et al.* (1972*a*), starting with *NN*-dimethyl-*p*-phenylenediamine. The compound coupled to the CNBr-activated Sepharose 4B, namely *p*-(6-aminohexanamido)phenyltrimethylammonium bromide hydrobromide, had m.p. 188–190°C and an absorption coefficient of $14\,900\,M^{-1}\cdot cm^{-1}$ at 243 nm in water. Dudai & Silman (1975) reported m.p. 191–194°C and an absorption coefficient of $15\,500\,M^{-1}\cdot cm^{-1}$ for this compound.

The MAC–Sepharose 4B gel was synthesized by the procedure of Dudai & Silman (1975), starting with 9-chloroacridine and 1,2-diaminopropane. The compound coupled to the CNBr-activated Sepharose 4B, namely 9-[2-(6-aminohexanamido)propylamino]-10-methylacridinium bromide hydrobromide had m.p. 255–257°C (decomp.) and an absorption coefficient of $12\,000\,M^{-1}\cdot cm^{-1}$ at 410 nm. Dudai & Silman (1975) reported m.p. 240°C (decomp.) and the same absorption coefficient.

Testing the retention of BuChE by affinity gels on a small scale

The capacities of the affinity gels for the BuChE enzymes in native sera and in partially purified serum fractions were estimated by using 1.0 ml gel columns (7 mm internal diam. × 26 mm high). The gel columns and partially purified samples of BuChE

were equilibrated with 0.10M-NaCl/50mM-sodium phosphate buffer, pH8.0. The ionic strength of native serum samples was not adjusted before passage through the columns. Sample solutions were passed through the gel columns at 4°C at 3 ± 0.2ml/h with the use of a Pharmacia P-3 pump. Column eluents were collected in fractions of volume 1–5ml and the BuChE activity in each fraction was determined.

Method I

The first method explored the possibility of using the procainamide–Sepharose 4B gels to treat the large volumes of serum needed to obtain adequate supplies of enzyme. Advantage was taken of the observation that both horse BuChE and human BuChE were retained by procainamide–Sepharose 4B gel columns when native serum was passed through them, although the capacity of the gel for the BuChE in horse serum was about 3 times that for the BuChE in human serum. The first step therefore involved passing either 3 litres of human or 9 litres of horse serum through 100ml columns of procainamide–Sepharose 4B gel. To improve the capacity of the gel for BuChE, the serum was first passed through a 500ml 'scrubber' column of DEAE-Sephadex A-50, run in tandem with the procainamide–Sepharose 4B gel column. The 'scrubber' column doubled the capacity of the procainamide–Sepharose 4B gel column for BuChE, presumably by removing proteins that tended to bind to the procainamide–Sepharose 4B gel and compete with the BuChE. Serum continued to be passed through the gel columns until retention of BuChE activity had fallen to about 90% of the applied activity. At this point the procainamide–Sepharose 4B gel held about 2g of protein, of which 40mg was BuChE. About 850mg of contaminant protein was washed off in the three rinses with solution containing up to 0.1M-NaCl in 50mM-sodium phosphate buffer, pH8.0. The BuChE was then eluted with a selective eluent, namely procainamide, as described below under 'Method II'. This was necesary to obtain maximum purification. The product, although purified over 2000-fold (Table 1), was only 20–25% BuChE. Passing the product a second time through a column of procainamide–Sepharose 4B gel was not effective. However, ion-exchange chromatography on DEAE-Sephadex with a choline chloride gradient (Main *et al.*, 1974) gave products that appeared to be electrophoretically pure. To ensure purity, the products from ion-exchange chromatography were subjected to preparative polyacrylamide-gel electrophoresis (Main *et al.*, 1972). This treatment did not change the specific activity of the horse BuChE product, but increased that of the human BuChE by 18% (Table 1).

Method II

In the second method, the procainamide–Sepharose 4B gels were used in the final step to obtain pure enzyme. A reasonably efficient method based on $(NH_4)_2SO_4$ fractionation had already been developed to treat the large volumes of serum required initially (Main *et al.*, 1974), and this was used as the first step. As it turned out, these two were the only steps needed. The $(NH_4)_2SO_4$ fractionation procedure had been developed with horse serum BuChE, and it had to be modified for use with human serum BuChE. The modifications were necesary because human BuChE is about 10 times less stable than horse BuChE in the pH3.0–4.0 range. In addition, human BuChE, unlike horse BuChE, is not precipitated quantitatively in the presence of 65%-saturated $(NH_4)_2SO_4$ at neutral pH. Method II was as follows.

Method II, step 1: $(NH_4)_2SO_4$ fractionation. The volumes of serum treated are given in Table 1. The $(NH_4)_2SO_4$ fractionation procedure described by Main *et al.* (1974) was followed with the following modification: (1) with the acid step, the pH was lowered to pH3.5 rather than to pH3.0, and the temperature was maintained at 20°C rather than at 24°C; (2) final precipitation of human BuChE was with 70%-saturated $(NH_4)_2SO_4$ at pH5.0. The precipitate was allowed to develop overnight before centrifugation.

Method II, step 2: procainamide–Sepharose 4B gel chromatography. The products from step 1 were dialysed against 50mM-sodium phosphate buffer, pH8.0, with sufficient changes to bring the product solutions to about the buffer concentration. The product solutions were then adjusted to 0.10M-NaCl and applied to 100ml (2.5cm × 20.5cm) procainamide–Sepharose 4B gel columns that had been equilibrated with 0.10M-NaCl/50mM-sodium phosphate buffer, pH8.0. All operations were at 4°C. After application of the sample, the columns were rinsed first with 700ml of 0.10M-NaCl/50mM-sodium phosphate buffer, pH8.0, and then with 400ml of 0.15M-NaCl/50mM-phosphate buffer. BuChE was eluted by applying a linear gradient of procainamide (0.25mM/ml) at the rate of 65ml/h. The gradient was developed with a Pharmacia P-3 pump, with a limit solution of 200ml of 0.10M-procainamide in 50mM-sodium phosphate buffer, pH8.0, and a starting solution of 200ml of 50mM-sodium phosphate buffer, pH8.0. The BuChE activity peaked at 17mM-procainamide. The eluate fractions containing the activity were pooled and dialysed against three successive 6-litre changes of 50mM-sodium phosphate buffer, pH8.0. After removal of samples for analysis, the bulk of the products were dialysed further against three successive 6-litre changes of distilled deionized water, freeze-dried to a powder and stored at −20°C.

Table 1. *Results of the procainamide–Sepharose-4B-gel-column procedures for purifying human BuChE and horse BuChE*

For details of the purification and other methods see the text.

Step	Vol. (ml)	Total activity (units)	Total protein (A_{280})	Yield overall (%)	Specific activity (units/mg)	Purification (fold)
Method I: Application of native sera to the procainamide gel in the first step						
Human BuChE						
Serum	5900*	16700	292000	100	0.057	1
Procainamide-gel chromatography	142	10600	82.3	63.7	129	2270
DEAE-Sephadex chromatography	59	7620	28.5	45.8	267	4680
Preparative polyacrylamide-gel electrophoresis	46.5	5970	18.9	35.8	315	5530
					479†	8400†
Horse BuChE						
Serum	9000	23000	492000	100	0.046	1
Procainamide-gel chromatography	84	17000	136	73.8	125	2690
DEAE-Sephadex chromatography	75	16100	35.1	70.1	459	9980
Preparative polyacrylamide-gel electrophoresis	45	13600	29.5	59.2	462	10000
					679†	14700†
Method II: Application of product from $(NH_4)_2SO_4$ fractionation to procainamide gel						
Human BuChE						
Serum	12500	35800	658000	100	0.054	1
$(NH_4)_2SO_4$ fractionation	2300	16600	26600	46	0.62	12
Procainamide-gel chromatography	92	12200	34.4	34	355	6500
					546†	10000†
Horse BuChE						
Serum	20000	64000	1210000	100	0.053	1
$(NH_4)_2SO_4$ fractionation	1100	38000	36500	60	1.04	20
Procainamide-gel chromatography	76	29500	63.3	46	466	8000
					684†	11000†

* From two gel runs, 2.95 litres/run.
† By dry weight.

Measurement of bound/free enzyme ratios for affinity gels

Equilibration of a cholinesterase or acetylcholinesterase with a slurry of affinity gel was done in a 1 ml-capacity Reacti-Vial from Pierce Chemical Co., Rockford, IL, U.S.A. Mixing was achieved by rotating the vials through 360°C at the rate of 10–12 turns/min. After equilibration the vials were centrifuged for 2 min at about 1500 rev./min in a International model CL clinical centrifuge equipped with a six-place swinging-bucket head. Centrifugation precipitated the gel, leaving a clear supernatant, which was analysed for free enzyme (e_f).

The enzyme stock solutions were in 50 mM-sodium phosphate buffer, pH 8.0, and had the following characteristics. Human BuChE stock solution contained 2.40 mg of protein/ml on the basis of an absorption coefficient of 1.52 $mg^{-1} \cdot ml \cdot cm^{-1}$ at 280 nm. The active-site concentration was 24.0 μM on the basis of an active-site relative mass of 100000. The specific activity was 471 units/mg of protein, with 1 mM-butyrylthiocholine as substrate. Horse BuChE stock solution contained 1.60 mg of protein/ml on the basis of an absorption coefficient of 1.45 $mg^{-1} \cdot ml \cdot cm^{-1}$ at 280 nm. The active-site concentration was 15.1 μM on the basis of an active-site relative mass of 106000. The specific activity was 660 units/mg of protein, with 1 mM-butyrylthiocholine as substrate. Electric-eel AcChE stock solution contained 2.36 mg of protein/ml on the basis of an absorption coefficient of 1.8 $mg^{-1} \cdot ml \cdot cm^{-1}$ at 280 nm (Rosenberry *et al.*, 1972). The activity was 240 units/ml with 1 mM-acetylcholine; the active-site concentration was 3.4 μM on the basis of titration of a portion of stock with Paraoxon. The specific activity was 102 units/mg of protein, with 1 mM-acetylthiocholine as substrate. Assuming an active-site relative mass of 100000, the preparation contained about 14% AcChE and 86% contaminant proteins.

Stock solutions of the various affinity gels were prepared by allowing 80 ml of a moderately concentrated gel slurry contained in a stoppered 100 ml graduated cylinder to settle for at least 2 weeks at

Table 2. *Comparison of binding of human BuChE, horse BuChE and electric-eel AcChE*

Relative binding ratios (e_b/e_f) of human BuChE, horse BuChE and electric-eel AChE to procainamide (PRO)–, MAC–, *m*-PTA– and *p*-PTA–Sepharose 4B affinity gels are given. Each binding ratio was measured at 5°C, in 50mM-sodium phosphate buffer, pH 8.0, with 110–120μg of enzyme preparation and 0.02ml of settled gel (except where noted) in a total volume of 0.5ml at the indicated NaCl concentration. Equilibration was for at least 16h before analysis. Comparisons with DEAE-Sephadex A-50 gel (DEAE-SX) were also made. The final enzyme concentrations are given in parentheses on the column headings.

Affinity-gel (μmol of ligand/ml of settled gel)	Relative binding ratio ($e_b/e_f = R$) Human BuChE (2.4μM)	Horse BuChE (2.3μM)	Electric-eel AcChE (0.34μM)
0M-NaCl			
PRO (22.9)	62	23	760
MAC (8.0)	2.0	0.2	1180
p-PTA (6.5)	0.45	0.15	21
m-PTA (6.0)	0.13	0.05	3.3
DEAE-SX	136	56	—
0.10M-NaCl			
PRO (22.9)	2.2	1.0	216
MAC (8.0)	0.40	0.15	328
p-PTA (6.5)	0.025	<0.01	0.90
m-PTA (6.0)	0.025	<0.01	0.28
DEAE-SX	0.10	0.045	—
0.20M-NaCl			
PRO (22.9)	0.32	0.27	—
0M-NaCl, 0.10ml of settled gel			
MAC (8.0)	9.2	0.47	—
p-PTA (6.5)	2.1	0.40	—
m-PTA (6.0)	0.6	0.10	—
DEAE-SX	520	129	—

5°C. The gels were in 50mM-sodium phosphate buffer, pH 8.0. The volume of packed gel was noted, and sufficient buffer was either added or withdrawn so that the settled gel was 40% of the total volume. The gels were thoroughly mixed with the buffer just before measured portions were withdrawn for experiments. Appropriate volumes of the various stock solutions were added to the Reacti-Vials to give a final volume of 0.50ml. Eight vials containing various combinations of affinity gels and cholinesterase or acetylcholinesterase were usually run together. The same affinity-gel stock solutions were used for all the values reported in Table 2.

Equilibration between bound and free enzyme was not instantaneous after the mixing of gel and enzyme, but required about 3h at 5°C for both human BuChE and horse BuChE in reaction with procainamide–Sepharose 4B gels, as shown in Fig. 1(*a*). At 25°C, equilibrium appeared to be reached in about 1h with horse BuChE (Fig. 1*a*). To ensure complete equilibration between free and gel-bound enzyme with the various gels used, mixing was allowed to continue 16h at 5°C before analysis.

Active-site relative masses by titration with di-isopropyl phosphorofluoridate and Paraoxon

The active-site relative masses of electrophoretically pure preparations of human BuChE and horse BuChE were determined as described previously (Main *et al.*, 1972, 1977), with BuChE preparations obtained by method I involving a final preparative polyacrylamide-gel-electrophoresis step. The results are given in the legend to Fig. 2, which includes the conditions used. Figs. 2(*a*) and 2(*b*) show the titration curves from which the intercept values were obtained and the range of di-isopropyl phosphorofluoridate and Paraoxon concentrations used.

The active-site relative masses obtained, 100000 and 106000 for human BuChE and horse BuChE respectively, are significantly higher than the monomeric subunit relative masses obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, which are about 85000 (e.g. Muensch *et al.*, 1976). The reason for this discrepancy is not known. Similar discrepancies have been reported for electric-eel AcChE by Dudai *et al.* (1972*a*), who gave a value of 107000 for the active-site relative mass and values of 88000 and 64000 for the relative masses of the polypeptide components. Muensch *et al.* (1976) reported a value close to 2 di-isopropyl phosphorofluoridate-binding sites per tetrameric molecule of human BuChE, and Lockridge & La Du (1978) reported '4 active sites/molecule'. Lockridge & La Du (1978) based their results on $A_{280}^{0.1\%} = 1.8$, referring to the absorption coefficient for AcChE reported by Rosenberry *et al.* (1972). When the absorption coefficient for human BuChE, determined by Haupt *et al.* (1966) and equal to 1.5, is substituted for the AcChE value, 3.3 active sites/molecule of human BuChE are indicated rather than 4. This value of 3.3 in turn gives an active-site relative mass of 108000. Main *et al.* (1974) previously reported an active-site relative mass of 103000 for horse BuChE, a value in good agreement with the 106000 now reported in the present paper.

Results and discussion

Large-scale purifications

The results obtained with the two methods with the use of procainamide–Sepharose 4B gels are compared in Table 1. Both methods gave electrophoretically pure preparations of human BuChE and horse BuChE, but, as mentioned, the methods did not work as well for purifying human BuChE.

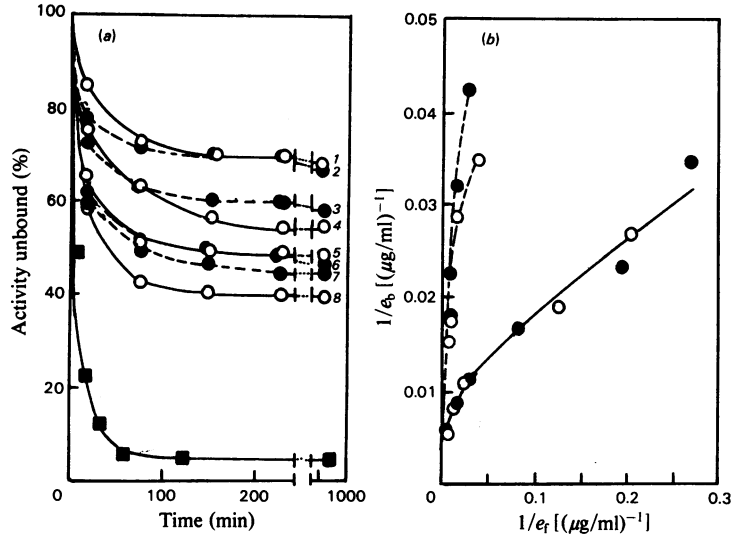

Fig. 1. *Time course to equilibrium (a) and maximum capacity (b) of procainamide–Sepharose 4B gels binding human BuChE and horse BuChE*

(*a*) Human BuChE (200μg) (○) or horse BuChE (188μg) (●) was mixed with 0.01ml of settled gel (curves *1* and *2*), 0.02ml of settled gel (curves *3* and *4*), 0.04ml of settled gel (curves *5* and *6*) and 0.06ml of settled gel (curves *7* and *8*) at 5°C, in 0.60ml of 0.10M-NaCl/50mM-sodium phosphate buffer, pH8.0, for various times as shown. The equilibration binding time curve of 89μg of horse BuChE and 0.04ml of settled gel at 25°C in 1.0ml of 50mM-sodium phosphate buffer, pH8.0, is also shown (■). (*b*) Double-reciprocal plot of free ($1/e_f$) to bound ($1/e_b$) enzyme from which the maximum capacity of the procainamide–Sepharose 4B gel for BuChE enzymes was estimated and the characteristics of binding were examined. The amount of gel was held constant (0.02ml of settled gel), and amounts of human BuChE (○) varying from 20 to 180μg or of horse BuChE (●) varying from 14 to 150μg were used in a final volume of 0.50ml and at two concentrations of NaCl, namely 0.10M (——) and 0.20M (– – – –). Each reaction mixture was in 50mM-sodium phosphate buffer, pH8.0, and equilibration was for at least 16h at 5°C.

With horse BuChE, method II was easier to carry out, but method I gave better yields (Table 1). With human BuChE, method I was much more difficult to carry out, and method II was therefore preferred. In addition, the specific activity of the human BuChE preparation obtained by method II was 14% higher than that of the product from method I.

The final products from both methods were electrophoretically pure. At loadings of 60μg of BuChE, Coomassie Blue staining for protein showed one predominant band and one or two relatively faint bands of lower mobility than the predominant band. Substrate staining with indoxylacetate showed all bands to be active. The slower-moving faint bands developed on standing and appeared to be aggregates of the major protein component.

Although the overall retention of BuChE by procainamide–Sepharose 4B gels was 95–97%, the yields on the procainamide–Sepharose-4B-gel steps of both methods were significantly lower. A considerable part of this loss, about 15%, could be accounted for by a change in the substrate kinetics resulting from the absence of the mildly activating proteins present in the starting materials. This loss was demonstrated by adding purified BuChE back to native serum. For example, the activity of a solution of pure horse BuChE in the absence of serum was 9.2units/ml, whereas in the presence of serum the activity increased to 10.8units/ml, or by 17%. The remaining losses were largely accounted for in the rinses, by the 'scrubber' columns (method I) and by fraction selection of eluted solutions.

Comparison of the retention of human BuChE and horse BuChE in native serum by various affinity gels

Our results confirmed that procainamide–Sepharose 4B gel is satisfactory for the small-scale purification of human BuChE. On a large scale, however, procainamide–Sepharose 4B gel worked much better for the purification of horse BuChE, and, as mentioned, its use for purifying human BuChE left much to be desired. The possibility that some other affinity gel might be more effective was therefore investigated. The gels studied in addition to procainamide–Sepharose 4B gel were the *m*-PTA– and *p*-PTA–Sepharose 4B gels and the MAC–Sepharose 4B gels used to purify acetylcholinesterase (Dudai & Silman, 1975). Small test gel columns were employed as described in the Experimental section.

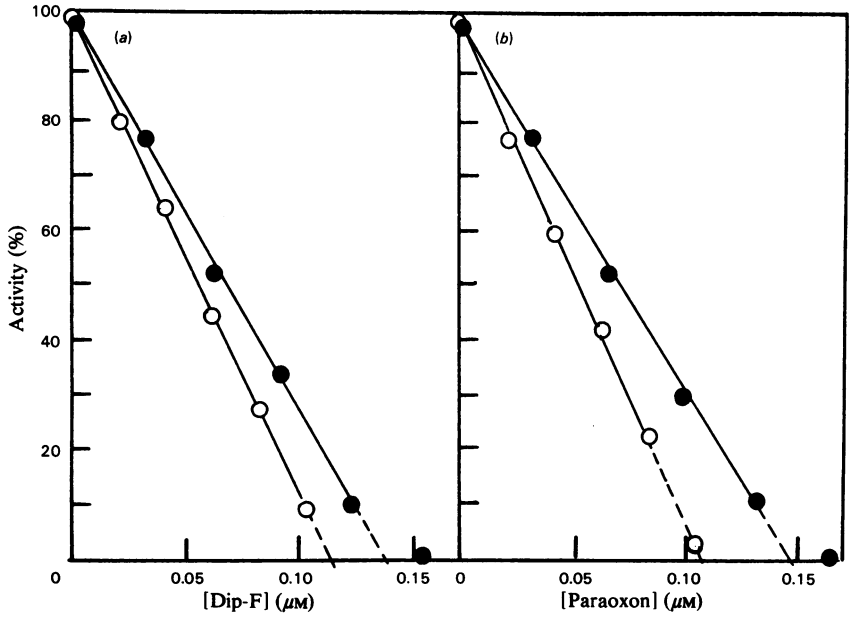

Fig. 2. *Active-site titration of human BuChE and horse BuChE with (a) di-isopropyl phosphorofluoridate and (b) Paraoxon*

Solutions containing either 0.0109mg of human BuChE/ml (○) or 0.0150mg of horse BuChE/ml (●) by dry weight were incubated with various concentrations of di-isopropyl phosphorofluoridate (Dip-F) (*a*) or Paraoxon (*b*) for 2h in 50mM-sodium phosphate buffer, pH8.0, at 25°C, at which time residual activities were determined with 1mM-butyrylthiocholine as substrate. Activities after longer incubations of at least 6h were also measured to ensure completion of inhibition. The active-site relative masses calculated from the intercepts at zero activity with di-isopropyl phosphorofluoridate were 96800 for human BuChE and 109000 for horse BuChE. Similarly, the active-site relative masses with Paraoxon were 103000 for human BuChE and 103000 for horse BuChE. The intercepts were determined by extrapolation of linear-regression fits to the points.

The results were plotted as retention profiles, and these indicated that, with human serum, the MAC–Sepharose 4B gel was superior to the procainamide–Sepharose 4B gel, with at least 4 times the capacity for human BuChE. In addition, the BuChE activity could be quantitatively recovered from the MAC–Sepharose 4B gel. For example, when 134 activity units in 47 bed volumes of human serum were applied to a 1ml MAC–Sepharose 4B gel, 122 units (91%) were retained. Of this, 120 units (or 98%) were washed off with a rinse of 2M-NaCl. MAC–Sepharose 4B gels may therefore be superior to procainamide–Sepharose 4B gels in purifying human BuChE.

In contrast, the retention profiles for horse serum BuChE indicated that procainamide–Sepharose 4B gels retain the BuChE in horse serum more effectively than did MAC–Sepharose 4B gels, although the difference was not great. Since the procainamide–Sepharose 4B gels have proved to be highly effective in purifying horse BuChE, and, since they are easier to synthesize than are MAC–Sepharose 4B gels, there would seem to be no advantage in using the latter to purify horse BuChE.

Comparison of bound/free enzyme ratios (R) of various affinity gels in combination with human BuChE, horse BuChE and electric-eel AcChE

The results from preliminary experiments indicated that the binding of BuChE enzymes to procainamide–Sepharose 4B gels could not be characterized by a single group K_d value. An example is the non-linear double-reciprocal plots of the concentration of free enzyme ($1/e_f$) against the concentration of bound enzyme ($1/e_b$) in Fig. 1(*b*). However, it seemed possible that values of $e_b/e_f = R$, obtained with uniform initial concentrations of enzyme and gel slurry, might provide a measure by which the binding potentials of the different affinity gels for the BuChE enzymes could be compared. The procedure described in the Experimental section was developed for this purpose, and the uniform conditions selected to determine R are given in the legend to Table 2. The amount of BuChE used (110–120μg/0.02ml of settled gel) was about half the amount needed to saturate the procainamide–Sepharose 4B gel. In general, the final conditions, such as volume, mixing time and amount of gel, were

based on the results from preliminary experiments [e.g. Figs. 1(*a*) and 1(*b*)].

The results with the four affinity gels and DEAE-Sephadex A-50 used are given in Table 2, and they suggest that the *R* values obtained provided a measure of binding with the cholinesterases and acetylcholinesterase with which valid comparisons could be made. With both BuChE enzymes, the *R* values characterizing the four gels were in the same order: procainamide–Sepharose 4B > MAC–Sepharose 4B > *p*-PTA–Sepharose 4B > *m*-PTA–Sepharose 4B in both 0M- and 0.10M-NaCl. Increasing the NaCl concentration from 0 to 0.10M decreased the procainamide–Sepharose-4B-gel *R* values by closely similar factors (approx. 25-fold) for both human BuChE and horse BuChE. With DEAE-Sephadex A-50, *R* decreased by about 1300-fold for both enzymes. In contrast, increasing the NaCl concentration decreased the *R* values for the MAC–Sepharose 4B gels by only 5-fold with human BuChE and 1.3-fold with horse BuChE. The effect of varying the salt concentration differed widely, depending on the type of gel, and was partly responsible for the selectivity of a given gel.

The *R* values characterizing the human BuChE were consistently higher (2–10-fold) than those of horse BuChE for all of the gels examined, including DEAE-Sephadex A-50. In particular, the *R* value characterizing the binding to procainamide–Sepharose 4B gel was 2.2-fold greater for human BuChE than for horse BuChE in 0.1M-NaCl. The relatively poor performance of the procainamide–Sepharose 4B gels at retaining the BuChE in native human serum was therefore not the result of poor binding by the human BuChE, but resulted from other causes. Experiments comparing the binding of human BuChE in 33%-saturated-$(NH_4)_2SO_4$ fractions obtained at either pH3.5 or pH4.0 indicated that human serum contained proteins that selectively bound to the procainamide–Sepharose 4B gel, excluding the BuChE. Proteins with comparable selectivity apparently are absent from horse serum, with which procainamide–Sepharose 4B gels were much more effective, despite the poorer binding of the horse BuChE.

This is paper no. 8415 of the Journal Series of the North Carolina Agricultural Research Service. The investigation was supported in part by Research Grant ES-00044 and Training Grant ES-07046 from the National Institute of Environmental Health Sciences.

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